THERAPY FOR HUMAN CANCERS USING CISPLATIN AND OTHER DRUGS OR GENES ENCAPSULATED INTO LIPOSOMES

Field of the Invention

5   The present invention relates to liposome encapsulated drugs and delivery systems, specifically liposome encapsulated cisplatin. The drugs are useful to kill cancer cells in a variety of human malignancies after intravenous injection.

Background of the Invention

10   Throughout this application various publications, patents and published patent specifications are referenced by author and date or by an identifying patent number. Full bibliographic citations for the publications are provided within this disclosure or immediately preceding the claims. The disclosures of these publications, patents and

15   published patent specifications are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

Cis-diamminedichloroplatinum(II), cis-$[Pt(NH_3)_2Cl_2]^{2+}$, abbreviated cisplatin or cis-DDP, is one of the most widely used antineoplastic drugs for the treatment of testicular, ovarian carcinomas and against carcinomas of the head and neck. More than

20   90% of testicular cancers are cured by cisplatin. The most severe side effects are nephrotoxicity, bone marrow toxicity and gastrointestinal irritation (Oliver and Mead, 1993). Bilateral optic neuropathy was observed in a patient affected by ovarian carcinoma treated with 160 mg/m^2 cisplatin and 640 mg/m^2 carboplatin (Caraceni et al., 1997). Oral hexamethylmelamine treatment in a group of 61 patients with epithelial

25   ovarian carcinoma with cis- or carboplatin resistance (relapse within 6 months after the end of that therapy) showed a 14% objective response rate (Vergote et al., 1992).

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Cationic cholesterol derivatives have been used to deliver therapeutic agents. For example, they have been mixed with phosphatidylethanolamine and sonicated to form small unilamellar vesicles which can complex with DNA and mediate the entry into the cytosol from the endosome compartment. One of the liposome formulations, DC-Chol liposomes, has been used in a gene therapy clinical trial against melanoma. Human immunodeficiency virus-1 transactivating protein gene was codelivered with a reporter gene under the control of HIV-1 long terminal repeat. Human tumor cells selected for cisplatin resistance or isolated from patients who had failed cisplatin therapy were highly transfectable with cationic liposomes. These results suggested a serial therapy protocol with cisplatin and gene therapy for malignancy (Farhood et al., 1994).

Various platinum complexes prepared from 2-amino-methylpyrrolidine derivatives as carrier ligands were tested for their antitumor activity against Colon 26 carcinoma and P388 leukemia using subcutaneous and/or intraperitoneal injections in mice. 2-aminomethylpyrrolidine proved to be the most effective carrier ligand in its amine derivatives (Morikawa et al., 1990).

An optimum procedure was established by orthogonal test for preparing cisplatin albumin microspheres (Cis-DDP-AMS) with emulsion-heating stabilization method (mean size was 148 microns). The distribution and elimination half times of platinum were prolonged 3.36 times and 1.23 times after hepatic arterial chemoembolization with Cis-DDP-AMS versus Cis-DDP, respectively (Zhang et al., 1995).

The search for platinum (II)-based compounds with improved therapeutic properties was prompted to design and synthesize a new family of water-soluble, third generation cis-diaminedichloroplatinum (II) complexes linked to uracil and uridine. However, none of the synthesized compounds showed any significant cytotoxic activity against three cell lines that were treated (Kim et al., 1998).

The recently developed bioreductive agent 4-[3-(2-nitroimidazolyl)-propylamino]-7-chloroquinoline hydrochloride (NLCQ-1) was found to potentiate the antitumor effect of the chemotherapeutic agents melphalan (L-PAM), cisplatin (cisDDP) and cyclophosphamide (CPM) without concurrent enhancement in bone marrow toxicity. Potentiation was strictly schedule dependent and the optimum effect (1.5 to 2 logs killing

beyond additivity) was observed when NLCQ-1 was given 45-min before cisDDP. These results support the classification of NLCQ-1, based on clinical studies, as a chemosensitizer (Papadopoulou et al., 1998).

A combination of paciltaxel with cisplatin as second-line treatment in patients with non-small cell lung cancer (NSCLC) who had previously undergone first-line therapy with cisplatin achieved partial response (40%) in 14 patients (Stathopoulos et al., 1999).

Abra et al. (U.S. Patent No. 5,945,122, issued August 31, 1999) describes a liposome composition containing entrapped non-charged cisplatin in mostly neutral lipids. However, the process of Abra et al. uses neutral lipids compared with the anionic lipid DPPG disclosed in the present patent for cisplatin entrapment.

Thus, while the prior reports indicate that liposome mediated delivery of cisplatin and other therapeutic drugs is possible, therapeutic efficiency has been limited by the low aqueous solubility and low stability of cisplatin. Therapeutic efficacy also is limited by the high toxicity of the drug. Thus, a need exists to reduce the difficulties involved in processing of cisplatin containing drugs and high toxicity of cisplatin when used therapeutically. This invention satisfies this need and provides related advantages as well.

Disclosure of the Invention

In one aspect, this invention provides a method for encapsulating cisplatin and other positively-charged drugs into liposomes having a different lipid composition between their inner and outer membrane bilayers and able to reach primary tumors and their metastases after intravenous injection to animals and humans. In one aspect, the method includes complex formation between cisplatin with DPPG (dipalmitoyl phosphatidyl glycerol) or other lipid molecules to convert cisplatin to its aqua form by hydrolysis which is positively-charged and is the active form of cisplatin endowed with the antineoplastic activity. At this stage membrane fusion peptides and other molecules with fusogenic properties may be added to improve entrance across the cell membrane of the complex. The aqua cisplatin-DPPG micelles are converted into liposomes by mixing

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with vesicle forming lipids such as pre-made liposomes or lipids followed by dialysis and extrusion through membranes, entrapping and encapsulating cisplatin to a very high yield. Doxorubicin or other positively-charged compounds can be substituted for cisplatin in these formulations. The encapsulated cisplatin has a high therapeutic efficacy

5   in eradicating a variety of solid human tumors including but not limited to breast carcinoma and prostate carcinoma. Combination of the encapsulated cisplatin with encapsulated doxorubicin or with other antineoplastic drugs are claimed to be of therapeutic value. Also of therapeutic value in cancer eradication are claimed to be combinations of encapsulated cisplatin with a number of anticancer genes including but

10 not limited to p53, IL-2, IL-12, angiostatin, and oncostatin encapsulated into liposomes as well as combinations of encapsulated cisplatin with HSV-tk plus encapsulated ganciclovir.

Brief Description of the Figures

15 Figure 1 depicts cisplatin encapsulation.

Figure 2 shows MCF-7 tumor regression in SCID mice after 3-4 injections of encapsulated cisplatin.

Figure 3 shows histology of tumors in SCID mice with or without treatment with cisplatin. Figure 3A shows untreated MCF-7 tumors grown in SCID mice. 40X

20 magnification. Notice the homogeneous pattern of structures characteristic of tumor tissue. Figure 3B shows cisplatin-treated mice (4 injections). Cells are apoptotic, there are groups of cells into structures and nuclei stain bigger and darker, characteristic of apoptotic cells. Figure 3C shows tumors from untreated animals showing invasion to muscle. 20X. Figure 3D shows cisplatin-treated mice. Invasion is not evident. 20X

25 magnification.

Figure 4 shows macroscopic (visual) difference in tumor size between an animal treated with encapsulated cisplatin (A) and an untreated animal (B)).

Modes for Carrying Out the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of immunology, molecular biology, microbiology, cell biology and recombinant DNA. These methods are described in the following publications. See, *e.g.,* Sambrook, et al. MOLECULAR CLONING: A LABORATORY MANUAL, 2nd

5 edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel, et al. eds., (1987)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.); "PCR: A PRACTICAL APPROACH" (M. MacPherson, et al., IRL Press at Oxford University Press (1991)); PCR 2: A PRACTICAL APPROACH (M.J. MacPherson, B.D. Hames and G.R. Taylor eds. (1995)); ANTIBODIES, A

10 LABORATORY MANUAL (Harlow and Lane, eds. (1988)); and ANIMAL CELL CULTURE (R.I. Freshney, ed. (1987)).

As used in the specification and claims, the singular form "a," "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

15 The term "comprising" is intended to mean that the compositions and methods include the recited elements, but not excluding others. "Consisting essentially of" when used to define compositions and methods, shall mean excluding other elements of any essential significance to the combination. Thus, a composition consisting essentially of the elements as defined herein would not exclude trace contaminants from the isolation

20 and purification method and pharmaceutically acceptable carriers, such as phosphate buffered saline, preservatives, and the like. "Consisting of" shall mean excluding more than trace elements of other ingredients and substantial method steps for administering the compositions of this invention. Embodiments defined by each of these transition terms are within the scope of this invention.

25 The terms "polynucleotide" and "nucleic acid molecule" are used interchangeably to refer to polymeric forms of nucleotides of any length. The polynucleotides may contain deoxyribonucleotides, ribonucleotides, and/or their analogs. Nucleotides may have any three-dimensional structure, and may perform any function, known or unknown. The term "polynucleotide" includes, for example, single-, double-stranded and triple

30 helical molecules, a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA,

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ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A nucleic acid molecule may also comprise modified nucleic acid molecules.

5   A "gene" refers to a polynucleotide containing at least one open reading frame that is capable of encoding a particular polypeptide or protein after being transcribed and translated.

A "composition" is intended to mean a combination of active agent and another compound or composition, inert (for example, a detectable agent or label or a

10   pharmaceutically acceptable carrier) or active, such as an adjuvant.

A "pharmaceutical composition" is intended to include the combination of an active agent with a carrier, inert or active, making the composition suitable for diagnostic or therapeutic use *in vitro, in vivo* or *ex vivo*.

As used herein, the term "pharmaceutically acceptable carrier" encompasses any

15   of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see Martin, REMINGTON'S PHARM. SCI., 15th Ed. (Mack Publ. Co., Easton (1975)).

20   An "effective amount" is an amount sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations, applications or dosages.

A "subject," "individual" or "patient" is used interchangeably herein, which refers to a vertebrate, preferably a mammal., more preferably a human. Mammals include, but

25   are not limited to, murines, simians, humans, farm animals, sport animals, and pets.

A "control" is an alternative subject or sample used in an experiment for comparison purpose. A control can be "positive" or "negative." For example, where the purpose of the experiment is to determine a correlation of an altered expression level of a gene with a particular type of cancer, it is generally preferable to use a positive control (a

30   subject or a sample from a subject, carrying such alteration and exhibiting syndromes

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characteristic of that disease), and a negative control (a subject or a sample from a subject lacking the altered expression and clinical syndrome of that disease).

A "gene delivery vehicle" is defined as any molecule that can carry inserted polynucleotides into a host cell. Examples of gene delivery vehicles are liposomes, cationic liposomes, viruses, such as baculovirus, adenovirus, adeno-associated virus, and retrovirus, bacteriophage, cosmid, plasmid, fungal vectors and other recombination vehicles typically used in the art which have been described for expression in a variety of eukaryotic and prokaryotic hosts, and may be used for gene therapy as well as for simple protein expression.

A "viral vector" is defined as a recombinantly produced virus or viral particle that comprises a polynucleotide to be delivered into a host cell, either *in vivo, ex vivo* or *in vitro*. Examples of viral vectors include retroviral vectors, adenovirus vectors, adeno-associated virus vectors and the like. In aspects where gene transfer is mediated by a retroviral vector, a vector construct refers to the polynucleotide comprising the retroviral genome or part thereof, and the inserted polynucleotide. As used herein, "retroviral mediated gene transfer" or "retroviral transduction" carries the same meaning and refers to the process by which a gene or nucleic acid sequences are stably transferred into the host cell by virtue of the virus entering the cell and integrating its genome into the host cell genome. The virus can enter the host cell via its normal mechanism of infection or be modified such that it binds to a different host cell surface receptor or ligand to enter the cell. As used herein, retroviral vector refers to a viral particle capable of introducing exogenous nucleic acid into a cell through a viral or viral-like entry mechanism.

Retroviruses carry their genetic information in the form of RNA; however, once the virus infects a cell, the RNA is reverse-transcribed into the DNA form, which integrates into the genomic DNA of the infected cell. The integrated DNA form is called a provirus.

In aspects where gene transfer is mediated by a DNA viral vector, such as an adenovirus (Ad) or adeno-associated virus (AAV), a vector construct refers to the polynucleotide comprising the viral genome or part thereof, and a polynucleotide to be inserted. Adenoviruses (Ads) are a relatively well characterized, homogenous group of

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viruses, including over 50 serotypes. (see, *e.g.,* WO 95/27071). Ads are easy to grow and do not require integration into the host cell genome. Recombinant Ad-derived vectors, particularly those that reduce the potential for recombination and generation of wild-type virus, have also been constructed. (see, WO 95/00655; WO 95/11984). Wild-type AAV has high infectivity and specificity integrating into the host cells genome. (Hermonat and Muzyczka (1984) PNAS USA **81**:6466-6470; Lebkowski, et al. (1988) Mol. Cell. Biol. **8**:3988-3996).

Vectors that contain both a promoter and a cloning site into which a polynucleotide can be operatively linked are well known in the art. Such vectors are capable of transcribing RNA *in vitro* or *in vivo*, and are commercially available from sources such as Stratagene (La Jolla, CA) and Promega Biotech (Madison, WI). In order to optimize expression and/or *in vitro* transcription, it may be necessary to remove, add or alter 5' and/or 3' untranslated portions of the clones to eliminate extra, potential inappropriate alternative translation initiation codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites can be inserted immediately 5' of the start codon to enhance expression.

Gene delivery vehicles also include several non-viral vectors, including DNA/liposome complexes, and targeted viral protein DNA complexes. Liposomes that also comprise a targeting antibody or fragment thereof can be used in the methods of this invention. To enhance delivery to a cell, the nucleic acid or proteins of this invention can be conjugated to antibodies or binding fragments thereof which bind cell surface antigens, *e.g.,* TCR, CD3 or CD4.

Polynucleotides are inserted into vector genomes using methods well known in the art. For example, insert and vector DNA can be contacted, under suitable conditions, with a restriction enzyme to create complementary ends on each molecule that can pair with each other and be joined together with a ligase. Alternatively, synthetic nucleic acid linkers can be ligated to the termini of restricted polynucleotide. These synthetic linkers contain nucleic acid sequences that correspond to a particular restriction site in the vector DNA. Additionally, an oligonucleotide containing a termination codon and an

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appropriate restriction site can be ligated for insertion into a vector containing, for example, some or all of the following: a selectable marker gene, such as the neomycin gene for selection of stable or transient transfectants in mammalian cells; enhancer/promoter sequences from the immediate early gene of human CMV for high

5   levels of transcription; transcription termination and RNA processing signals from SV40 for mRNA stability; SV40 polyoma origins of replication and ColE1 for proper episomal replication; versatile multiple cloning sites; stabilizing elements 3' to the inserted polynucleotide, and T7 and SP6 RNA promoters for *in vitro* transcription of sense and antisense RNA. Other means are well known and available in the art.

10   "Host cell" is intended to include any individual cell or cell culture which can be or have been recipients for vectors or the incorporation of exogenous polynucleotides, polypeptides and/or proteins. It also is intended to include progeny of a single cell, and the progeny may not necessarily be completely identical (in morphology or in genomic or total DNA complement) to the original parent cell due to natural., accidental., or

15   deliberate mutation. The cells may be prokaryotic or eukaryotic, and include but are not limited to bacterial cells, yeast cells, plant cells, insect cells, animal cells, and mammalian cells, *e.g.*, murine, rat, simian or human.

As used herein, the terms "neoplastic cells," "neoplasia," "tumor," "tumor cells," "cancer" and "cancer cells," (used interchangeably) refer to cells which exhibit relatively

20   autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation (i.e., de-regulated cell division). Neoplastic cells can be malignant or benign.

"Suppressing" tumor growth indicates a growth state that is curtailed when compared to control cells. Tumor cell growth can be assessed by any means known in

25   the art, including, but not limited to, measuring tumor size, determining whether tumor cells are proliferating using a ^{3}H-thymidine incorporation assay, or counting tumor cells. "Suppressing" tumor cell growth means any or all of the following states: slowing, delaying, and stopping tumor growth, as well as tumor shrinkage.

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Embodiments of the Invention

Micelles, Liposomes and Processes for Obtaining Them

Claimed herein is a new method for entrapping cisplatin into lipids which

5 enhances the content of cisplatin per volume unit, reduces its toxicity, is able to target

primary tumors and their metastases after intravenous injection, and shows shrinkage of

tumors and complete therapy of SCID mice bearing human tumors.

Cisplatin is a heavy metal complex containing two chloride atoms and two amino

groups in the cis position attached to one atom of the transitory heavy metal platinum in

10 its divalent form. It is a bifunctional alkylating agent as well as DNA intercalator

inhibiting DNA synthesis. In one form, cisplatin is a yellow powder of a molecular

weight of 300.1 and of limited solubility of 1 mg/ml in water. It is widely used for the

treatment of cancer patients, especially those of testicular, lymphomas, endometrial,

bladder, ovarian, head and neck squamous cell carcinomas, breast carcinomas, and many

15 other malignancies, often in combination with adriamycin, vinblastin, bleomycin,

prednisone, vincristine, taxol, and others antineoplastic drugs as well as radiation therapy.

We claim reduction in total cisplatin volume required for patient treatment because of an

increase in its solubility in its lipid entrapment form.

The volume used for intravenous injection is usually large (about 180 ml per adult

20 patient or about 20-120 mg/m^2) administered as a 24-hour infusion. It is cleared from the

plasma in a rapid phase of 25-80 min followed by a slower secondary phase of 58-73 h; it

is bound by plasma proteins and excreted by the kidneys (explaining the severe kidney

toxicity in treated patients). Dose related nephrotoxicity can be partially overcome with

vigorous hydration, mannitol, furosemide and other drugs. Other toxicities incurred by

25 cisplatin include ototoxicity, nausea and vomiting, anemia, and mild myelosuppression

(The Merck Manual of Diagnosis and Therapy). The present invention overcomes the

limitations of prior art processes and compositions.

Thus, in one aspect, this invention provides methods for producing cisplatin

micelles, by combining cisplatin and a phosphatidyl glycerol lipid derivative (PGL

30 derivative) in a range of 1:1 to 1:2.1 to form a cisplatin mixture. In alternative

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embodiments, the range of cisplatin to PGL derivative is in the ranges 1:1.2; or 1:1.4; or 1:1.5; or 1:1.6; or 1:1.8 or 1:1.9 or 1:2.0 or 1:2.1. The mixture is then combined with an effective amount of at least a 20% organic solvent such as an ethanol solution to form cisplatin micelles.

5   As used herein, the term "phosphatidyl glycerol lipid derivative (PGL derivative)" is any lipid derivative having the ability to form micelles and have a net negatively charged head group. This includes but is not limited to dipalmitoyl phosphatidyl glycerol (DPPG), dimyristoyl phosphatidyl glycerol, and dicapryl phosphatidyl glycerol. In one aspect, phosphatidyl derivatives with a carbon chain of 10 to 28 carbons and having

10   unsaturated side aliphatic side chain are within the scope of this invention. The complexation of cisplatin with negatively-charged phosphatidyl glycerol lipids having variations in the molar ratio giving the particles a net positive (1:1) neutral (1:2) or slightly negative (1:2.1)charge will allow targeting of different tissues in the body after administration. However, complexing of cisplatin with negatively charged PGL has been

15   shown to enhance the solubility of cisplatin, thus reducing the volume of the drug required for effective antineoplastic therapy. In addition, the complexation of cisplatin and negatively charged PGL proceeds to very high encapsulation efficiency minimizing drug loss during the manufacturing process. These complexes are stable, do not form precipitates and retain therapeutic efficacy after storage at 4°C for at least 4 months.

20   As used herein, the term "cisplatin" included analogs. Examples include carboplatin, ormaplatin, oxaplatin, 2-aminomethylpyrrolidine (1,1-cyclobutane dicarboxylato)platinum, lobaplatin, 1-cyclobutane-dicarboxylato(2-)-(2-methyl-1,4-butanediamine-N,N')platinum, zeniplatin, enloplatin, 254-S nedaplatin and JM-216 (bis-acetato-amine-dichloro-cyclohexylamine-platinum(IV)).

25   It is to be understood, although not always explicitly stated, that other positively charged drugs, including but not limited to the antineoplastic drug doxorubicin can be substituted for cisplatin. Alternatively, other types of drugs that are neutral can be used upon their conversion into positively charged drugs by derivation with positively charged groups. Modification of a neutral or negatively-charged anticancer or other type of drug

30   to a positively-charged molecule can be accomplished by a number of methods well

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established in organic synthesis. This can be achieved for example by replacing a hydroxyl group in the drug with an amino group or by a trimethylamino group thus introducing a positive charge to the compound. Replacement of a ring hydroxyl group with an amino group is discussed in United States Patent 5,837,868, Wang, et al. isssued

5   November 17, 1998.

The above method does not require that the steps be performed in the order indicated above. For example, the method can be practiced by combining cisplatin with an effective amount of at least a 20% organic solvent solution to form a solution. The solution is combined with a phosphatidyl glycerol lipid (PGL) derivative in a range of 1:1

10   to 1:2.1 to form a cisplatin micelle. As above, the range of cisplatin to PGL derivative is in the ranges 1:1.2; or 1:1.4; or 1:1.5; or 1:1.6; or 1:1.8 or 1:1.9 or 1:2.0 or 1:2.1.

Any organic solvent or formulation of ethanol, or any other alcohol that does not form a two phase system, or other organic solvent (i.e., choroform), that is miscible in 20% alcohol, is useful in the methods described herein. For example, the alcohol solution

15   can be any of at least 30%, 35%, 40%, 45% up to 90%, including any increment in between. Preferably, the alcohol solution is 30% ethanol for DPPG, and for other lipids the optimal percentage may be different.

In one embodiment, partial replacement of DPPG molecules by peptides with a net negative charge gives to cisplatin complexes having fusogenic properties able to cross

20   the cell membrane of the target. Fusogenic peptides may also be covalently attached at the free and of PEG for their better exposure. Addition of a small amount of cationic lipids replacing positive charges of aqua cisplatin at the final cisplatin/DPPG complex also endows the complex with fusogenic properties; the percentage of positive charges to be substituted by cationic lipids (*e.g.,* DDAB, dimethyldioctadecyl ammonium bromide;

25   DMRIE: N-[1-(2,3-dimyristyloxy)propyl]-N,N-dimethyl-N-(2-hydroxyethyl) ammonium bromide; DMTAP: 1,2-dimyristoyl-3-trimethylammonium propane; DOGS: Dioctadecylamidoglycylspermine; DOTAP: N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride; DPTAP: 1,2- dipalmitoyl-3-trimethylammonium propane; DSTAP: 1,2-disteroyl-3-trimethylammonium propane) is small because of the toxicity of

cationic lipids. Some formulations contain an amount of the fusogenic amphiphilic lipid DOPE in the micelles.

In a further aspect, the cisplatin micelles are encapsulated into vesicle forming lipids, for example, for use in drug delivery.

5   The lipid encapsulated cisplatin has a high therapeutic efficacy in eradicating a variety of solid human tumors including but not limited to breast carcinoma, prostate carcinoma, glioblastoma multiform, non-small lung cell carcinoma, pancreatic carcinoma, head and neck squamous cell carcinoma and T-cell lymphomas. Accordingly in another aspect, the invention provides a method for the treatment of a variety of human

10   malignancies using an encapsulated cisplatin or alternatively, other positively-charged antineoplastic drugs into liposomes having a different lipid composition between their inner and outer membrane bilayers. The liposome encapsulated drugs are able to reach primary tumors and their metastases after intravenous injection to animals and humans.

A combination of the encapsulated cisplatin with doxorubicin or with other

15   antineoplastic drugs have a higher therapeutic efficacy than cisplatin alone. Also of higher therapeutic efficacy in cancer eradication are combinations of encapsulated cisplatin with a number of anticancer genes including but not limited to p53, IL-2, IL-12, angiostatin, and oncostatin encapsulated into similar type of liposomes as well as combinations of encapsulated cisplatin with HSV-tk plus encapsulated ganciclovir.

20   In a further aspect, the invention provides a combination of the encapsulated cisplatin with the following genes:

(i) A wild-type (wt) p53 cDNA expression vector under control of the CMV, beta-actin, or other promoters, and human origins of replication able to sustain long term expression of the p53 gene; viral origins of replication which require viral replication

25   initiator proteins such as T antigen for their activation are nor suitable for the transfer of the p53 gene because p53 protein interacts strongly with T antigen.

(ii) A PAX5 cDNA expression vector, the only suppressor of the p53 gene known (both of the wt and mutant p53 genes) interacting with a short (10 nucleotide) regulatory region within intron 1 of the p53 gene. A major drawback in p53 gene therapy is the

30   inactivation of the wt p53 protein by the endogenous mutated forms of p53 which are

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overexpressed in tumors and which are able to tetramerize with wt p53 protein; the endogenous p53 genes will be suppressed by expression of Pax5, a potent transcriptional repressor of the p53 gene. The wt p53 cDNA vector lacks intron 1 and by consequence the suppressive PAX5 binding region. It is important to suppress the endogenous mutant

5   p53 gene expression and eliminate mutant p53 from the cancer cells to potentiate induction of apoptosis and tumor suppression.

(iii) The herpes simplex virus thymidine kinase (HSV-tk) gene. The herpes simplex virus thymidine kinase (HSV-tk) suicide gene will be also included in combinations of p53 and Pax5 genes causing interruption in DNA synthesis after

10   ganciclovir (GCV) treatment of the animal model and human patient; this is expected to increase the strand breaks in the cancer cells and to potentiate the tumor suppressor functions of p53 known to bind to strand breaks and to damaged DNA sites. In a further embodiment, ganciclovir is combined and encapsulated into liposomes.

Gene therapy is a new era of biomedical research aimed at introducing therapeutic

15   genes into somatic cells of patients (reviewed by Boulikas, 1998a; Martin and Boulikas, 1998). Two major obstacles prohibit successful application of somatic gene transfer: (1) the small percentage of transduced cells and (2) the loss of the transcription signal of the therapeutic gene after about 3-7 days from injection in vivo.

The first problem arises (i) from inability of delivery vehicles carrying the gene to

20   reach the target cell surface (the vast majority of liposome-DNA complexes are eliminated from blood circulation rapidly); (ii) from difficulty to penetrate the cell membrane and (iii) to release the DNA from endosomes after internalization by cells; (iv) from inefficient import into nuclei. Others have used stealth liposomes (Martin and Boulikas, 1998a), which persist in circulation for days and concentrate in tumors.

25   However, classical stealth liposomes are not taken up by cancer cells. Disclosed herein are strategies that are designed to enhance liposome internalization (fusogenic peptides).

The second problem results from the loss of the plasmids in the nucleus by nuclease degradation and failure to replicate autonomously leading to their dilution during cell proliferation among progeny cells or by inactivation of the foreign DNA after

30   integration into the chromosomes of the host cell. However, the use of human sequences

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able to sustain extrachromosomal replication of plasmids for prolonged periods (see U.S. Patent on "Cloning method for trapping human origins of replication" by Teni Boulikas No. 5,894,060) will overcome this limitation.

Also claimed herein are tumor regression and reduction in tumor mass volume of breast, prostate and other cancers in animal models and in humans after delivery of encapsulated cisplatin (termed Lipoplatin™) or encapsulated doxorubicin, and combinations of these drugs with genes including but not limited to the p53, PAX5, and HSV-tk/encapsulated ganciclovir, IL-2, IL-12, GM-CSF, angiostatin, IL-4, IL-7, IFN-gamma, TNF-alpha, RB, BRCA1, E1A, cytosine deaminiase in combination with encapsulated 5-fluorocytosine, bcl-2, MDR-1, p21, p16, bax, bcl-xs, E2F, IGF-I VEGF, TGF-beta genes and the like.

Accordingly, this invention also provides a method for delivering cisplatin or other therapeutic agent to a cell comprising contacting the cell with the encapsulated drugs or other intermediate, obtainable by the methods of this invention. Also provided by this invention is a method for inhibiting the growth of a tumor in a subject, comprising administering to the subject an effective amount of the encapsulated drugs obtainable by the methods of this invention. The methods can be practiced *in vitro*, *ex vivo* or *in vivo*.

Thus, this invention also provides combination therapy using encapsulated drugs and polynucleotides. As used herein, a polynucleotides includes but is not limited to genes encoding proteins and polypeptides as well as sequences encoding ribozymes and antisense. The combination therapy is more effective in eradicating cancer than either treatment alone because the two mechanisms are different and can achieve a synergism. For example, the property of p53 protein to bind to damaged DNA regions and free ends of DNA is known and also to trigger the mechanism of apoptosis in severely-damaged cells (reviewed by Boulikas, 1998a). Free ends of DNA in cancer cells are expected to be produced after damage by cisplatin enhancing the induction of an apoptotic pathway in these cells by the expression of the transferred wt p53 (many tumors have mutated p53 and might be unable to induce effectively this pathway). The therapeutic polynucleotide also can be inserted into a gene transfer vector prior to incorporation into the micelle.

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Transfer of the wild-type p53 gene has been successfully used to slow-down tumor cell proliferation in vivo and in cell culture in numerous studies. Intratumoral injection using adenoviral/p53 vectors has been shown to be effective against lung tumors in recent clinical trials (Roth et al., 1996) and against prostate tumors on animal models

5 (reviewed by Boulikas, 1998a). The intratumoral injection method, however, may not be applicable to metastases often associated with late stages of cancer. Systemic delivery of the p53 gene with encapsulated cisplatin and targeting of tumors in any region of the body is an effective treatment for cancer cure. We claim strategies for ameliorating or partially overcoming the four main obstacles for successful somatic gene transfer using

10 liposomal delivery of the wt p53, pax5, HSV-tk, GM-CSF, IL-12, IL-2, IL-4, IL-7, IFN-gamma, TNF-alpha, RB, BRCA1, E1A, cytosine deaminiase in combination with encapsulated 5-fluorocytosine, bcl-2, MDR-1, p21, p16, bax, bcl-xs, E2F, IGF-I VEGF, TGF-beta, angiostatin and other genes in combination with encapsulated cisplatin and other drugs to a variety of human cancers in animal models and in patients to be tested in

15 clinical trials. These include: (i) concentration and encapsulation of the drug and gene bullets into liposomes reducing their toxicity; (ii) targeting of solid tumors and metastases by coating of the surface of the complexes with polyethylene glycol (PEG), hyaluronic acid or other polymers; (iii) enhancement in uptake of drugs and plasmids by cancer cells because of the fusogenic peptides or small percentage of cationic lipids, and (iv)

20 sustained expression of the genes using human origins of replication (ORIs) able to sustain episomal replication or long term expression of therapeutic genes and high levels of expression.

In a further embodiment, the polynucleotides or genes further comprise regulatory DNA sequences that sustain expression of the genes for months rather than days. This

25 translates into fewer treatments and less suffering of the cancer patient. It can also exert a strong therapeutic effect because of higher levels of expression of the anticancer gene; the same gene placed under control of weak regulatory DNA will be ineffective.

Several experimental strategies for cancer treatment have been designed using p53 gene delivery; our novelty consists in that the endogenous mutant p53 forms, which are

30 overexpressed in over half of human prostate malignancies especially those from

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advanced prostate cancer are suppressed using the PAX5 expression vector. Mutated forms of p53 have amino acid substitutions mainly in their DNA binding domain but are still able to tetramerize with the wt p53 form; p53 acts as a tetramer and the presence of high levels of endogenous mutant p53 in human cancers cells interferes with the tumor

5   suppressor functions of the wt p53 to be delivered.

PAX5 is an homeodomain protein which determines body structures during development; PAX5 is expressed at early stages of mammalian development and in the adult during differentiation in hematopoietic stem cells; p53 gene expression is eliminated by the PAX5 suppressor protein at early stages of development allowing cells

10   to multiply fast in the developing embryo. PAX5 is switched off at later stages throughout adulthood allowing p53 to be expressed and exert its tumor suppressive functions and to regulate apoptosis especially in the hematopoietic cell lineage.

A number of delivery systems are being used in somatic gene transfer, each associated with advantages and drawbacks. Recombinant adenoviruses do not replicate

15   efficiently; recombinant murine retroviruses integrate randomly and are inactivated by chromatin surroundings; recombinant AAV integrates randomly and cannot achieve high titers for clinical utility. All have a maximum capacity of 3.5-7.5 kb of foreign DNA because of packaging limitations. Naked DNA is rapidly degraded (half-life 5 minutes) after systemic delivery. Cationic liposomes are toxic do not survive in circulation beyond

20   a heart beat and target mainly the endothelium of the lung, liver, and heart. So far, only "stealth" liposomes have been proven capable of concentrating in tumor sites (also in liver and spleen) and to survive for prolonged periods in blood circulation (*e.g.*, one day compared with minutes for non-stealth neutral liposomes and a few seconds for cationic liposomes). However, stealth liposomes are not taken readily by tumor cells remaining in

25   the extracellular space where they release their load over days after lysis (reviewed by Martin and Boulikas, 1998); however, the aspect of the invention described below modifies stealth liposomes with fusogenic peptides or by providing a partially cationic lipid composition or DOPE at their inner bilayer which would endow them to enter the tumor cell membrane by causing disturbance of the lipid bilayer.

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Having attained concentration and uptake of the drug and gene bullets in solid tumors in animals with stealth liposomes, the second step is efficacy of our drug and gene targeting approach. A human clinical trial at M.D. Anderson Cancer Center uses transfer of the wild-type p53 gene in patients suffering with non-small cell lung cancer and shown

5   to have p53 mutations in their tumors using local injection of an Ad5/CMV/p53 recombinant adenovirus at the site of tumor in combination with cisplatin. The first results of this clinical trial are encouraging after intratumor injection of p53 (Roth et al., 1996; reviewed by Boulikas, 1998a). However, local injection is not applicable to metastases often associated with advanced stages of malignancies; in particular, prostate

10   cancer gives metastases to bones by a mechanism involving stimulation in prostate tumor proliferation by insulin-like growth factor I (IGF-I) which is especially secreted by bone cells. Therefore, the delivery system proposed here, able to concentrate into the tumor cell mass after systemic injection, is likely to treat not only the primary tumor but also its metastases.

15   The proposed cisplatin liposomes will primarily target tumors because of the nature of our delivery system. The genes in the combination therapy will primarily target dividing cells because of the use of HSV-tk and ganciclovir that incorporates into replicating DNA, and primarily vascularizing tumors because of the use of stealth liposomes. Thus, liver and spleen cells that are also reached by stealth liposomes will not

20   be killed.

In a further embodiment, the liposome encapsulated drugs described herein further comprise an effective amount of a fusogenic peptide. Fusogenic peptides belong to a class of helical amphipathic peptides characterized by a hydrophobicity gradient along the long helical axis. This hydrophobicity gradient causes the tilted insertion of the

25   peptides in membranes, thus destabilizing the lipid core and, thereby, enhancing membrane fusion (Decout et al., 1999).

Hemagglutinin (HA) is a homotrimeric surface glycoprotein of the influenza virus. In infection, it induces membrane fusion between viral and endosomal membranes at low pH. Each monomer consists of the receptor-binding HA1 domain and the

30   membrane-interacting HA2 domain. The NH2-terminal region of the HA2 domain

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(amino acids 1 to 127), the so-called "fusion peptide," inserts into the target membrane and plays a crucial role in triggering fusion between the viral and endosomal membranes. Based on substitution of eight amino acids in the region 5-14 with cysteines and spin-labeling electron paramagnetic resonance it was concluded that the peptide forms an

5   alpha-helix tilted approximately 25 degrees from the horizontal plane of the membrane with a maximum depth of 15 angstroms (Å) from the phosphate group (Macosko et al., 1997). Use of fusogenic peptides from influenza virus hemagglutinin HA-2 enhanced greatly the efficiency of transferrin-polylysine-DNA complex uptake by cells; in this case the peptide was linked to polylysine and the complex was delivered by the transferrin

10   receptor-mediated endocytosis (reviewed by Boulikas, 1998a). This peptide had the sequence: GLFEAIAGFIENGWEGMIDGGGYC (SEQ ID NO:1) and was able to induce the release of the fluorescent dye calcein from liposomes prepared with egg yolk phosphatidylcholine which was higher at acidic pH; this peptide was also able to increase up to 10-fold the anti-HIV potency of antisense oligonucleotides, at a concentration of

15   0.1-1 mM, using CEM-SS lymphocytes in culture. This peptide changes conformation at the slightly more acidic environment of the endosome destabilizing and breaking the endosomal membrane (reviewed by Boulikas, 1998a).

The presence of negatively charged lipids in the membrane is important for the manifestation of the fusogenic properties of some peptides but not of others; whereas the

20   fusogenic action of a peptide, representing a putative fusion domain of fertilin, a sperm surface protein involved in sperm-egg fusion, was dependent upon the presence of negatively charged lipids. However, that of the HIV2 peptide was not (Martin and Ruysschaert, 1997).

For example, to analyze the two domains on the fusogenic peptides of influenza

25   virus hemagglutinin HA, HA-chimeras were designed in which the cytoplasmic tail and/or transmembrane domain of HA was replaced with the corresponding domains of the fusogenic glycoprotein F of Sendai virus. Constructs of HA were made in which the cytoplasmic tail was replaced by peptides of human neurofibromin type 1 (NF1) (residues 1441 to 1518) or c-Raf-1, (residues 51 to 131). The constructs were expressed in CV-1

30   cells by using the vaccinia virus-T7 polymerase transient-expression system. Membrane

fusion between CV-1 cells and bound human erythrocytes (RBCs) mediated by parental or chimeric HA proteins showed that, after the pH was lowered, a flow of the aqueous fluorophore calcein from preloaded RBCs into the cytoplasm of the protein-expressing CV-1 cells took place. This indicated that membrane fusion involves both leaflets of the

5   lipid bilayers and leads to formation of an aqueous fusion pore (Schroth-Diez et al., 1998).

A remarkable discovery was that the TAT protein of HIV is able to cross cell membranes (Green and Loewenstein, 1988) and that a 36-amino acid domain of TAT, when chemically crosslinked to heterologous proteins, conferred the ability to transduce

10   into cells. It is worth mentioning that the 11-amino acid fusogenic peptide of TAT (YGRKKRRQRRR (SEQ ID NO:2)) is a nucleolar localization signal (see Boulikas, 1998b).

Another protein of HIV, the glycoprotein gp41, contains fusogenic peptides. Linear peptides derived from the membrane proximal region of the gp41 ectodomain

15   have potential applications as anti-HIV agents and inhibit infectivity by adopting a helical conformation (Judice et al., 1997). The 23 amino acid residues N-terminal peptide of HIV-1 gp41 has the capacity to destabilize negatively charged large unilamellar vesicles. In the absence of cations the main structure was a pore-forming alpha-helix, whereas in the presence of $Ca2^+$ the conformation switched to a fusogenic, predominantly extended

20   beta-type structure. The fusion activity of HIV(ala) (bearing the R22(A substitution) was reduced by 70% whereas fusogenicity was completely abolished when a second substitution (V2(E) was included arguing that it is not an alpha-helical but an extended structure adopted by the HIV-1 fusion peptide that actively destabilizes cholesterol-containing, electrically neutral membranes (Pereira et al., 1997).

25   The prion protein (PrP) is a glycoprotein of unknown function normally found at the surface of neurons and of glial cells. It is involved in diseases such as bovine spongiform encephalopathy, and Creutzfeldt-Jakob disease in the human, where PrP is converted into an altered form (termed PrPSc). According to computer modeling calculations, the 120 to 133 and 118 to 135 domains of PrP are tilted lipid-associating

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peptides inserting in a oblique way into a lipid bilayer and able to interact with liposomes to induce leakage of encapsulated calcein (Pillot et al., 1997b).

The C-terminal fragments of the Alzheimer amyloid peptide (amino acids 29-40 and 29-42) have properties related to those of the fusion peptides of viral proteins

5   inducing fusion of liposomes in vitro. These properties could mediate a direct interaction of the amyloid peptide with cell membranes and account for part of the cytotoxicity of the amyloid peptide. In view of the epidemiologic and biochemical linkages between the pathology of Alzheimer's disease and apolipoprotein E (apoE) polymorphism, examination of the potential interaction between the three common apoE isoforms and the

10 C-terminal fragments of the amyloid peptide showed that only apoE2 and apoE3, not apoE4, are potent inhibitors of the amyloid peptide fusogenic and aggregational properties. The protective effect of apoE against the formation of amyloid aggregates was thought to be mediated by the formation of stable apoE/amyloid peptide complexes (Pillot et al., 1997a; Lins et al., 1999).

15 The fusogenic properties of an amphipathic net-negative peptide (WAE 11), consisting of 11 amino acid residues were strongly promoted when the peptide was anchored to a liposomal membrane; the fusion activity of the peptide appeared to be independent of pH and membrane merging and the target membranes required a positive charge which was provided by incorporating lysine-coupled phosphatidylethanolamine

20 (PE-K). Whereas the coupled peptide could cause vesicle aggregation via nonspecific electrostatic interaction with PE-K, the free peptide failed to induce aggregation of PE-K vesicles (Pecheur et al., 1997).

A number of studies suggest that stabilization of an alpha-helical secondary structure of the peptide after insertion in lipid bilayers in membranes of cells or

25 liposomes is responsible for the membrane fusion properties of peptides; Zn^{2+}, enhances the fusogenic activity of peptides because it stabilizes the alpha-helical structure. For example, the HEXXH domain of the salivary antimicrobial peptide, located in the C-terminal functional domain of histatin-5, a recognized zinc-binding motif is in a helicoidal conformation (Martin et al., 1999; Melino et al., 1999; Curtain et al., 1999).

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Fusion peptides have been formulated with DNA plasmids to create peptide-based gene delivery systems. A combination of the YKAKnWK peptide, used to condense plasmids into 40 to 200 nm nanoparticles, with the GLFEALLELLESLWELLLEA (SEQ ID NO:3) amphipathic peptide, which is a pH-sensitive lytic agent designed to facilitate

5   release of the plasmid from endosomes enhanced expression systems containing the beta-galactosidase reporter gene (Duguid et al., 1998).

DOPE (dioleyl phosphatidyl ethanolamine) is a fusogenic lipid; elastase cleavage of N-methoxy-succinyl-Ala-Ala-Pro-Val-DOPE converted this derivative to DOPE (overall positive charge) to deliver an encapsulated fluorescent probe, calcein, into the

10   cell cytoplasm (Pak et al., 1999). An oligodeoxynucleic sequence of 30 bases complementary to a region of beta-endorphin mRNA elicited a concentration-dependent inhibition of beta-endorphin production in cell culture after it was encapsulated within small unilamellar vesicles (50 nm) containing dipalmitoyl-DL-alpha-phosphatidyl-L-serine endowed with fusogenic properties (Fresta et al., 1998).

15   Additional fusogenic peptides useful in the methods of this invention are described in Table 1 below.

Fusogenic peptide	Source Protein	Properties	Reference
GLFEAIAGFIENG WEGMIDGGGYC	Influenza virus hemagglutinin HA-2		Bongartz et al, 1994;
YGRKKRRQRRR	TAT of HIV		Green and Loewenstein, 1988;
the 23-residue fusogenic N-terminal peptide	HIV-1 transmembrane glycoprotein gp41	Was able of inserting as an alpha-helix into neutral phospholipid bilayers	Curtain et al, 1999
120 to 133 and 118 to 135 domains	prion protein	tilted lipid-associating peptide; interact with liposomes to induce leakage of encapsulated calcein	Pillot et al, 1997b
29-42-residue fragment	Alzheimer's beta-amyloid peptide	Endowed with capacities resembling those of the tilted fragment of viral fusion proteins	Lins et al, 1999
nonaggregated amyloid beta-peptide (1-40)	Alzheimer's beta-amyloid peptide	induces apoptotic neuronal cell death	Pillot et al, 1999
LCAT 56-68 helical segment	lecithin cholesterol acyltransferase (LCAT)	forms stable beta-sheets in lipids	Peelman et al, 1999; Decout et al, 1999
70 residue peptide (SV-117)	Fusion peptide and N-terminal heptad repeat of Sendai virus	Induced lipid mixing of egg phosphatidylcholine/phosphatid yiglycerol (PC/PG) large unilamellar vesicles (LUVs)	Ghosh and Shai, 1999

Fusogenic peptide	Source Protein	Properties	Reference
MSGTFGGILAGL IGLL	N-terminal region of the S protein of duck hepatitis B Virus (DHBV)	Was inserted into the hydrophobic core of the lipid bilayer and induced leakage of internal aqueous contents from both neutral and negatively charged liposomes	Rodriguez-Crespo et al, 1999
MSPSSLLGLLAG LQVV	S protein of woodchuck hepatitis B virus (WHV)	Was inserted into the hydrophobic core of the lipid bilayer and induced leakage of internal aqueous contents from both neutral and negatively charged liposomes	Rodriguez-Crespo et al, 1999
peptide sequence B18	membrane-associated sea urchin sperm protein bindin	Triggers fusion between lipid vesicles; a histidine-rich motif for binding zinc, is required for the fusogenic function	Ulrich et al, 1999
	histatin-5 (salivary antimicrobial peptide)	Aggregates and fuses negatively charged small unilamellar vesicles in the presence of Zn^{2+}	Melino et al, 1999
amphipathic negatively charged peptide consisting of 11 residues (WAE)		Forms an alpha-helix inserted and anchored into the membrane (favored at 37°C) oriented almost parallel to the lipid acyl chains; promotes fusion of large unilamellar liposomes (LUV)	Martin et al, 1999
A polymer of polylysine (average 190) partially substituted with histidyl residues		histidyl residues become cationic upon protonation of the imidazole groups at pH below 6.0.; disrupt endosomal membranes	Midoux and Monsigny, 1999
GLFEALLELLESL WELLLEA		amphipathic peptide; a pH-sensitive lytic agent to facilitate release of the plasmid from endosomes	Duguid et al, 1998

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Fusogenic peptide	Source Protein	Properties	Reference
(LKKL)$_4$		amphiphilic fusogenic peptide, able to interact with four molecules of DMPC	Gupta and Kothekar, 1997
residues 53-70 (C-terminal helix)	apolipoprotein (apo) AII	induces fusion of unilamellar lipid vesicles and displaces apo AI from HDL and r-HDL	Lambert et al, 1998
residues 90-111	PH-30 alpha (a protein functioning in sperm-egg fusion)	membrane-fusogenic activity to acidic phospholipid bilayers	Niidome et al, 1997
N-terminus of Nef	Nef protein of human immunodeficiency type 1 (HIV-1)	membrane-perturbing and fusogenic activities in artificial membranes; causes cell killing in E. coli and yeast	Macreadie et al, 1997
casein signal peptides	alpha s2- and beta-casein	Interact with dimyristoylphosphatidyl-glycerol and -choline liposomes; show both lytic and fusogenic activities	Creuzenet et al, 1997
amino-terminal sequence F1 polypeptide	F1 polypeptide of measles virus (MV)	Can be used as a carrier system for CTL epitopes	Partidos et al, 1996
23 hydrophobic amino acids in the amino-terminal region	S protein of hepatitis B virus (HBV)	A high degree of similarity with known fusogenic peptides from other viruses.	Rodriguez-Crespo et al, 1994

Fusogenic peptide	Source Protein	Properties	Reference
19-27 amino acid segment	glycoprotein gp51 of bovine leukemia virus	Adopts an amphiphilic structure and plays a key role in the fusion events induced by bovine leukemia virus.	Voneche et al, 1992
Ac-(Leu-Ala-Arg-Leu)$_3$-NHCH$_3$	basic amphipathic peptides	caused a leakage of contents from small unilamellar vesicles composed of egg yolk phosphatidylcholine and egg yolk phosphatidic acid (3:1)	Suenaga et al, 1989; Lee et al, 1992
amphiphilic anionic peptides E$_5$ and E$_5$L		can mimic the fusogenic activity of influenza hemagglutinin(HA)	Murata et al, 1991
30-amino acid peptide with the major repeat unit Glu-Ala-Leu-Ala (GALA)$_7$	designed to mimic the behavior of the fusogenic sequences of viral fusion proteins	becomes an amphipathic alpha-helix as the pH is lowered to 5.0 ; fusion of phosphatidylcholine small unilamellar vesicles induced by GALA requires a peptide length greater than 16 amino acids	Parente et al, 1988
pardaxin	amphipathic polypeptide, purified from the gland secretion of the Red Sea Moses sole flatfish Pardachirus marmoratus	forms voltage-gated, cation-selective pores; mediated the aggregation of liposomes composed of phosphatidylserine but not of phosphatidylcholine	Lelkes and Lazarovici, 1988
Gramicidin (linear hydrophobic polypeptide)		Antibiotic; induces aggregation and fusion of vesicles	Massari and Colonna, 1986; Tournois et al, 1990
poly(Glu-Aib-Leu-Aib) (Aib represents 2-aminoisobutyric acid),		Amphiphilic structure upon the formation of alpha-helix; caused fusion of EYPC liposomes and dipalmitoylphosphatidylcholine liposomes more strongly with decreasing pH	Kono et al, 1993

After the micelles have been formed, they are mixed with an effective amount of a vesicle forming lipid to form drug containing liposomes. Useful lipids for this invention

include premade neutral liposomes, lipids in powder, PEG-DSPE or hydrogenated soy phosphatidylchline (HSPC). Vesicle-forming lipids are selected to achieve a specified degree of fluidity or rigidity of the final complex providing the lipid composition of the outer layer. These can be composed of 10-60% cholesterol and the remaining amounts

5   include bipolar phospholipids, such as the phosphatidylcholine (PC) or phosphatidylethanolamine (PE), with a hydrocarbon chain length in the range of 14-22, and saturated with one or more double C=C bonds. A preferred lipid for use in the present invention is cholesterol (10-60%), hydrogenated soy phosphatidylcholine (HSPC) at 40-90%, and the derivatized vesicle-forming lipid PEG-DSPE at 1-7%. The liposomes

10   provide the outer lipid bilayer surfaces that are coated with the hydrophilic polymer, PEG. The PEG chains have a molecular weight between 1,000-5,000 Dalton. Other hydrophilic polymers include hyaluronic acid, polyvinylpyrrolidone, DSPE, hydroxyethylcellulose, and polyaspartamide. PEG-DSPC and PEG-HSPC are commercially available from Syngena.

15   Prior to mixture with the vesicle forming lipid, the ethanol or other organic solvent can be removed by any method known in the art, e.g., dialysis of the micelles through permeable membranes.

Diagnostic and Therapeutic Methods

20   We claim the therapy of subject, *e.g.*, mammals such as mice, rats, simians, and human patients, with human cancers including, but not limited to breast, prostate, colon, non-small lung, pancreatic, testicular, ovarian, cervical carcinomas, head and neck squamous cell carcinomas. In one aspect, intravenous injection of cisplatin encapsulated into liposomes as well as by combinations of encapsulated cisplatin with encapsulated

25   doxorubicin, fluorodeoxyuridine, bleomycin, adriamycin, vinblastin, prednisone, vincristine, taxol or radiation therapy, encapsulated oligonucleotides, ribozymes endowed with anticancer properties and a number of anticancer genes including but not limited to p53/Pax5/HSV-tk genes, are claimed. Our approach consists of two major parts: (i) the ability to target cancer cells (ii) effectiveness of our approach to kill cancer cells.

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Accordingly, this invention also provides a method for delivering cisplatin or other therapeutic agent to a cell comprising contacting the cell with the encapsulated drugs obtainable by the methods of this invention. Also provided by this invention is a method for inhibiting the growth of a tumor in a subject, comprising administering to the

5   subject an effective amount of the encapsulated drugs obtainable by the methods of this invention. Depending on the composition of the lipid/micelle formulation, also claimed herein are methods for targeting solid tumors and metastases in a subject by intravenous administration of an effective amount of the encapsulated drug and methods for penetrating the cell membrane of a tumor in a subject by administration of an effective

10   amount of the encapsulated drug, wherein the micelle contains a free fusogenic peptide or a fusogenic peptide-lipid conjugate.

The methods can be practiced in vitro, ex vivo or in vitro.

In vitro practice of the method involves removal of a tumor biopsy or culturing of a cell sample containing tumor cells. The final liposome complex or any intermediate

15   product arising during cisplatin encapsulation (e.g., micelles shown in Figure 1A) are contacted with the cell culture under conditions suitable for incorporation of the drug intracellularly. The in vitro method is useful as a screen to determine the best drug therapy for each individual patient. Inhibition of cell growth or proliferation indicates that the cell or tumor is suitably treated by this therapy. Effective amount of drug for

20   each therapy varies with the tumor being treated and the subject being treated. Effective amounts can be empirically determined by those of skill in the art.

When delivered to an animal, the method is useful to further confirm efficacy of the drug or therapy for each tumor type. As an example of suitable animal models, groups of SCID mice or nude mice (Balb/c NCR nu/nu female, Simonsen, Gilroy, CA)

25   may be subcutaneously inoculated with about 10^5 to about 10^9 cancer or target cells as defined herein. When the tumor is established, the liposome is administered.

As used herein, "administration, delivered or administered" is intended to include any method which ultimately provides the drug/liposome complex to the tumor mass. Examples include, but are not limited to, topical application, intravenous administration,

30   parenteral administration or by subcutaneous injection around the tumor. Tumor

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measurements to determine reduction of tumor size are made in two dimensions using venier calipers twice a week.

For in vivo administration, the pharmaceutical compositions are preferably administered parenterally, i.e., intravenously, intraperitoneally, subcutaneously,

5   intrathecally, injection to the spinal cord, intramuscularly, intraarticularly, portal vein injection, or intratumorally. More preferably, the pharmaceutical compositions are administered intravenously or intratumorally by a bolus injection. In other methods, the pharmaceutical preparations may be contacted with the target tissue by direct application of the preparation to the tissue. The application may be made by topical, "open" or

10   "closed" procedures. By "topical", it is meant the direct application of the pharmaceutical preparation to a tissue exposed to the environment, such as the skin, nasopharynx, external auditory canal, eye, inhalation to the lung, genital mucosa and the like. "Open" procedures are those procedures which include incising the skin of a patient and directly visualizing the underlying tissue to which the pharmaceutical preparations are applied.

15   This is generally accomplished by a surgical procedure, such as a thoracotomy to access the lungs, abdominal laparotomy to access abdominal viscera, or other direct surgical approach to the target tissue. "Closed" procedures are invasive procedures in which the internal target tissues are not directly visualized, but accessed via inserting instruments through small wounds in the skin. For example, the preparations may be administered to

20   the peritoneum by needle lavage. Likewise, the pharmaceutical preparations may be administered to the meninges or spinal cord by infusion during a lumbar puncture followed by appropriate positioning of the patient as commonly practiced for spinal anesthesia or metrazamide imaging of the spinal cord. Alternatively, the preparations may be administered through endoscopic devices.

25   Administration *in vivo* can be effected in one dose, continuously or intermittently throughout the course of treatment. Methods of determining the most effective means and dosage of administration are well known to those of skill in the art and will vary with the composition used for therapy, the purpose of the therapy, the target cell being treated, and the subject being treated. Single or multiple administrations can be carried out with

30   the dose level and pattern being selected by the treating physician. Suitable dosage

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formulations and methods of administering the agents can be empirically determined by those of skill in the art.

The agents and compositions of the present invention can be used in the manufacture of medicaments and for the treatment of humans and other animals by administration in accordance with conventional procedures, such as an active ingredient in pharmaceutical compositions.

Ideally, the drug/lipid formulation should be administered to achieve peak concentrations of the active compound at sites of disease. This may be achieved, for example, by the intravenous injection of the drug/lipid formula. Desirable blood levels of the drug may be maintained by a continuous infusion to provide a therapeutic amount of the active ingredient within disease tissue. The use of operative combinations is contemplated to provide therapeutic combinations requiring a lower total dosage of each component drugs than may be required when each individual therapeutic compound or drug is used alone, thereby reducing adverse effects.

While it is possible for the drug/lipid formula to be administered alone, it is preferable to present it as a pharmaceutical formulation comprising at least one active ingredient, as defined above, together with one or more pharmaceutically acceptable carriers therefor and optionally other therapeutic agents. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient.

Designing the third generation of vehicles for the delivery of anticancer drugs and genes to solid tumors at as described herein was the result of five major improvements over existing technologies:

1.) Encapsulation of antineoplastic drugs into sterically stabilized liposomes has reduced manifold their toxicity. This is anticipated to bring to an end the nightmare of cancer patients subject to chemotherapy. Most antineoplastic drugs under current use have severe side effects such as hair loss, vomiting, weight loss and cause infarction as well as damage to kidneys, brain, liver and all other vital tissues. The antineoplastic drugs described herein are hidden inside the lumen of the lipid bilayer, are not visible to most tissues and concentrate into their tumor targets, not in every tissue in the body.

Upon their uptake by the solid tumor they exert a specific cytotoxic effect to cancer cells without damaging normal cells.

2.) Targeting of solid tumors and their metastases all over the body. Over 95% of cancer patients succumb from complications connected to metastases, not from the primary tumor. Our gene and drug delivery system has been designed to evade the immune system after intravenous administration of the gene and drug bullet reaching not only the primary tumor but also every metastasis in the animal and human body regardless of the size of the tumor. It is based on the long circulation time of our drug and gene carrying vehicles and their extravasation through the vascular endothelium of tumors because of its imperfections and leakiness at its initial stage of formation (neoangiogenesis in growing tumors) as well as because of differences in hydrostatic pressure between the growing solid tumor and normal body tissues. The liposomes of this invention have a different composition between their inner and outer lipid layers permitting efficient encapsulation and tumor targeting.

3.) Uptake of the liposome bullet by the cancer cell. The liposome bullets are able to promote fusion with the cell membrane. Similar "stealth" bullets developed elsewhere are unable to cross the membrane barrier of the cancer cell.

4.) Reaching nearly 100% liposome encapsulation efficiency for anticancer drugs, oligonucleotides and genes is a major advancement. This means minimal loss and cost effective use of drugs and genes. It also translated into simpler steps in manufacturing the anticancer bullet.

5.) The unique technology described herein can identify regulatory DNA sequences that sustain expression of the genes in the anticancer bullet for months rather than days. This translates into fewer treatments and less suffering of the cancer patient. It can also exert a strong therapeutic effect because of higher levels of expression of the anticancer gene; the same gene placed under control of weak regulatory DNA will be ineffective.

The following examples are intended to illustrate, and not limit the invention.

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<u>Examples</u>

Preparation of Micelles and Lipid-Encapsulated Cisplatin

One formula for encapsulation includes the steps of: (A) mixing cisplatin (in

5   powder or other form) with DPPG (dipalmitoyl phosphatidyl glycerol) or other

negatively-charged lipid molecules at a 1:1 to 1:2 molar ratio in at least a 30% ethanol,

0.1 M Tris HCl, pH 7.5 to achieve about 5 mg/ml final cisplatin concentration.

Variations in the molar ratio between cisplatin and DPPG are also of therapeutic value

targeting different tissues. (B) Heating at 50° C. During steps A and B the initial powder

10   suspension, which tends to give a precipitate of the yellow cisplatin powder, is converted

into a gel (colloidal) form; during steps A and B there is conversion of cisplatin to its

aqua form (by hydrolysis of the chloride atoms and their replacement by water molecules

bound to the platin) which is positively-charged and is the active form of cisplatin

endowed with the antineoplastic activity; the aqua cisplatin is simultaneously complexed

15   with the negatively-charged lipid into micelles in 30% ethanol. This cisplatin-DPPG

electrostatic complex has already improved properties over free cisplatin in tumor

eradication. (C) The properties of the complex (and of the final formulation after step D,

see below) in passing through the tumor cell membrane after reaching its target are

improved by addition of peptides and other molecules that give to the complex this

20   property. (D) The cisplatin-DPPG micelle complex is converted into liposomes

encapsulating the cisplatin-DPPG monolayer (Figure 1 top) or to other type of complexes

by direct addition of premade liposomes followed by dialysis against saline and extrusion

through membranes to downsize these to 100-160 nm in diameter (Figure 1 bottom). It is

the lipid composition of added liposomes that determines the composition of the outer

25   surface of our final cisplatin formulation.

Variations in step (A) permit encapsulation of doxorubicin and other positively

charged antineoplastic compounds. Addition of positively charged groups to neutral or

negatively-charged compounds allows their encapsulation similarly into liposomes.
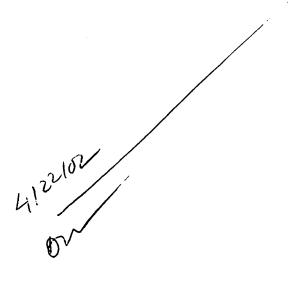
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Therapeutic Application

Ninety (90) day-release estrogen pellets were implanted subcutaneously into SCID female mice. The mice were subcutaneously injected at mammary fat pad with 7.5 million MCF-7 (a human breast carcinoma available from the ATCC) cells in 0.1 ml PBS. After establishment of tumors, the mice were injected intravenously at tail vein with 0.1 ml of cisplatin liposomes. Results are shown in Figures 2 to 4.

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

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